Bioaccumulation of metals in sediment elutriates and their effects on growth, condition index, and metallothionein contents in oyster larvae

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BIOACCUMULATION OF METALS IN SEDIMENT ELUTRIATES AND THEIR EFFECTS ON GROWTH, CONDITION INDEX AND METALLOTHIONEIN CONTENTS OF OYSTER LARVAE.

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Abstract: The bioavailability of Cd, Cu, Zn and Pb from two metal-contaminated sediment (Bidassoa and Dunkerque) was studied using *Crassostrea gigas* larvae exposed to sediment elutriates. The metal contents within the sediments, the larvae and larval growth, the condition index, and the induction of metallothionein in the larvae were measured. The larval growth and condition index were only affected after exposure to the highest elutriate concentration (5 to 25%) from the most contaminated sediment (Dunkerque). Bioaccumulation of all metals was observed in larvae exposed to Dunkerque elutriatre and only Cu bioaccumulation was observed in the case of Bidassoa elutriate. The compilation of results from larvae exposed to both sediment elutriate show a strongly correlation between bioaccumulated metal considered individually or combined and metallothionein level in larvae presenting no detrimental effect. In contrary, in the case of larvae exposed to the highest Dunkerque elutriate concentration and showing the highest metal body burdens, we observed a drop of the metallothionein level. These results indicate that metallothionein is a more sensitive indicator of heavy metal pollution, compared to physiological endpoints taking into account in bioassays and could be proposed as precocious biomarker of metals exposure in larvae. However, it must be take care to the "fault control" due to toxicological effect on larvae metabolism in the case of important contaminant exposure.

INTRODUCTION

Marine and coastal areas are constantly subjected to the introduction of natural and anthropogenic pollutants, which are mostly adsorbed by suspended particles and subsequently accumulated in the sediments. Sediments can accumulate metals at concentrations 10,000 times higher than in the overlying water column (Förstner 1979), constituting an important source of contamination and risk for living organisms. Coastal and estuarine areas serve as reproductive and nursery grounds for many invertebrate and fish species and should be preserved.

Benthic and epibenthic species are the most exposed to contaminants of sedimentary origin, to those which are adsorbed on sedimentary particles as well as to those which are dissolved in interstitial water. Pelagic organisms are equally exposed to sediment-bound contaminants either via the food web and/or after the remobilization of contaminants into the sea water (Chapman and Long 1983, Fichet et al. 1998, Miller et al. 2000). The contamination of water column occurs by diffusion and when the sediments are resuspended by natural factors such as bioturbation (Burgess et al. 1993, Peterson et al. 1996, Ciarelli et al. 1999, 2000), storms, wave and tide action and by human activities, such as dredging operations in particular (Chapman et al. 1998). When sediments are resuspended, the amounts of heavy metals, released into the sea water, are relatively low (Kwan and Dutka 1996, Van den berg et al. 2001) and do not generally induce short term toxicity. Long-term toxicity assessment of these low contamination levels requires development of sensitive and early warning biological tools. Elutriate tests allow to assess the toxicity of sedimentary metals released in water column when sediments are resuspended (Thompson et al. 1999).

Chemical analyses allow to characterize the contamination level of the medium (water or sediment), but they are inadequate to assess the biological quality of a studied zone. Only

living systems are able to integrate the various complex effects of contaminants that are really bio-available (Chapman and Long 1983). Numerous bioassays, standardized to varying degrees, have been developed during the two last decades (see Review Burton 1992; His et al. 1999). Bivalve embryo is one of the most sensitive organisms and has been used in numerous environmental toxicology studies (Taylor 1978, Carr et al. 1996, McPherson and Chapman 2000). Instead of the endpoints considered in bioassays, larval growth test is most difficult to perform but it is more sensitive (Geffard et al. 2002a) and allows to assess the bioavailability and toxicity of both soluble contaminant fraction and contaminants adsorbed on suspended particles (Geffard et al. 2002a).

Bioassays allow evaluating the toxicity of contaminants that are really bio-available and therefore constitute a useful tool to study the biological quality of estuaries and coastal areas. However, physiological endpoints of these tests are not early-warning biological responses. When deleterious effects are observed, the biological quality of the studied area is not good and the contamination levels have already marked and irreversible effects on living organisms. Moreover, bioassays do not allow identifying the compounds inducing the observed biological effects.

The bio-availability of contaminants depends on several factors such as physico-chemical properties of the contaminant itself, the characteristics of the environment and that of the organism used in the bioassay (Borgmann 2000). One of the recognized methods to assess the bio-availability of contaminants is to observe their accumulation in the test organisms. This method takes into account all factors that control their bioavailability (Connell et al. 1999, Borgmann 2000). Recently, for several compounds, some authors have determined critical body burden concentrations above which biological effects could be observed. However, this approach cannot be used with essential metals (Cu, Zn) and metabolized compounds such as PAHs.

Biomarkers could also be used to study the bio-availability of contaminants, such as metallothionein for metal exposures. Many studies have shown that metallothionein, a low-molecular-weight cytosolic protein, is induced by metal contamination in numerous taxa, including mammals, fish (Hamza-Chaffai et al. 1997) and marine invertebrates (Bebianno and Serafim 1998, Bebianno et al. 2000, Mouneyrac et al. 2000, 2002). It is generally admitted that the primary role of metallothionein is the homeostasis of essential metals such as Cu and Zn, but this protein is also involved in the detoxification of non-essential metals such as Ag, Cd and Hg (Roesijadi 1992, Amiard et al, 2005). Metallothionein-like protein has also been detected in bivalve larvae of *Mytilus galloprovincialis* (Pavicic et al. 1994) and *Crassostrea virginica* (Ringwood and Brouwer 1993, 1995, Roesijadi et al. 1997). However, the inductions are observed for studies using metallic salts and experimental doses unrealistic compared to environmental concentrations present even in metal-rich areas. More recently, metallothionein-like protein induction was detected in veligers of *M. galloprovincialis* exposed to natural contaminated sediment (Geffard et al. 2002c). However, these authors used freeze-dried sediment known to be more toxic than the fresh sediment (Geffard et al. 2002b, 2004).

The aim of this study was to use the larval growth test and the metallothionein concentration as a sensitive and early warning tool to assess the bioavailability and the toxicity of metals released into the water column when sediments are re-suspended. For this, fertilized eggs and larvae of *Crassostrea gigas* were exposed to elutriate obtained from natural sediments. Two metal rich sediments have been selected, one from a coastal zone (Bidassoa Estuary, French-Spanish border) and the second from the port of Dunkerque (North of France). The final aim of this study was to establish links between biochemical response (metallothionein), metal accumulation, larval growth and the condition index of larvae.

Materials and Methods.

*Sampling and conservation of sediment***.**

Sediments were sampled in July 1999. At the Bidassoa estuary, only the top 2 cm of the surface were scraped using a plastic blade. Sediments from the port of Dunkerque were collected with a stainless steel Van Veen grab. All sediments were wet-sieved at 2 mm to eliminate debris, homogenized and stored in glass bottles at 4°C in darkness for less than one week prior to bioassays.

Elutriate preparation.

Elutriates were prepared using a modification of Melzian's method (1990). Sediments were shaken mechanically (multi-wirst shaker, 500 rpm) in filtered seawater (FSW) at a ratio of 1:4 (sediment: water) for 8 h and allowed to settle for another 8 h before recovery of the supernatant (elutriate). Elutriate was diluted with FSW to concentrations of 0 (control), 5, 10, 25 and 50% for Bidassoa and 0 (control), 1, 5, 10 and 25% for Dunkerque. These concentrations have been selected because they do not previously induced abnormal effects on the embryonic development of *Crassostrea gigas*. The maximum concentrations tested correspond to the No Observed Effect Concentrations (NOEC; Geffard et al. 2002b).

Larval rearing.

Mature oysters were collected in Arcachon Bay (French) which is extensively used for oysters farming based on Pacific oysters *Crassostrea gigas* and was thus assumed to have a good 'biological quality' (Geffard et al. 2002b). The procedure to obtain embryos was described in detail by Geffard et al (2001a, 2002c). Fertilized eggs were counted and placed in 2-liter bakers (60,000 l^{-1}) filled with the different media to be tested (3 replications per treatment). After the first 24 h, veligers maintained in rearing status were placed in 2-liter beakers (10,000 larvae l^{-1}, 3 replications) and fed with *Isochrysis galbana* (150 algae μl^{-1}). All experimental solutions were renewed at 48-h intervals, using elutriates prepared some hours

before. At this time, larvae were photographed with a Canon camera fitted to an inverted microscope and larval height of 50 individuals per replicate (distance between the umbo and the ventral valve margin, Galtsoff 1964) was measured. The growth test was stopped after 10 days for Bidassoa elutriate. On contrary, Dunkerque elutriate had severe effects on *C. gigas* growth, so much so that experiments were respectively stopped on day 5 for the highest concentration and on day 7 for another. At the end, larvae were recovered through a sieve (32µm), rinsed with 0.9% aqueous ammonium formiate (to eliminate the salt), freeze-dried, weighed and stored at 4° C in hermetic bag before being used for analysis. Condition index (CI) of larvae from each replicate was determined using the following equation:

$$CI = \frac{\text{mean weight of a lyophilised larvae}\,(\mu g)}{\text{mean shell height of a larvae}\,(\mu m)} * 100$$

Pre-treatment of sediments for metal analysis.

Aliquots (3 replicates of 0.5 g) of each fresh sediment were taken from the well-homogenized total sample and placed in acid-washed glass tubes. These samples were then dried and weighed to determine metal concentration as a function of dry weight. Hot mineralisation (95°C) was performed by addition of 5 ml of HNO_3 and 3 ml of HCl. This process was conducted until dryness, and the residues were then suspended again in 10 ml of 1N HCl for metal analysis.

Extraction of metals and metallothionein from larvae.

Geffard et al (2002c) descrived the process for tissular compartmentalization of metals and partial purification of metallothionein. Each replication of lyophilized larvae was homogenized in Tris-NaCl buffer and the Cytosolic (S1) and insoluble (P1) fractions were separated by initial centrifugation (25,000 g, 55 min at 4°C). The insoluble fraction is constituted by all cellular and tissular debris plus shells of larvae. Metallothionein was isolated from an aliquot (50µl) of the S1 fraction by a second centrifugation (15,000g, 10 min at 4°C) after being subjected to heat (75°C, 15 min). This second supernatant (S2) containing

metallothionein was frozen at –80°C prior to be used for metallothionein analysis. Before metal analysis, an acid digestion step was required for soluble (S1) and insoluble (P1) fractions, using a procedure described in Geffard et al (2002c). After acid digestion, the solution obtained were supplemented to a known volume (2 ml) with deionised water. The three replications of each exposure condition were treated separately with the exception of the larvae exposed to 5, 10 and 25% of Dunkerque elutriate, where larval growth and the weight of larvae was low. The three replicate samples were mixed together to prevent problems of sensitivity related to the analytical tools.

Metal assays.

Following the acid digestion phase (for sediment and cytosolic (S1) and insoluble (P1) fraction of larvae), metals were analyzed by flame atomic absorption spectrophotometry (AAS) for Cu in sediment and Zn in sediment and larvae fraction. We used electrothermal AAS with Zeeman effect (Hitachi Z8200) for Cu in larvae fractions and for Cd and Pb in larvae fractions and sediment. The analytical method was previously described by Amiard et al (1987). To eliminate matrix effect, standard addition analysis was performed in an iso-medium, and the concentration of each element were +125, 250 and 500 ng ml^{-1} for Zn and Cu in flame AAS and +12.5, 25 and 50 ng ml^{-1} for Cu, +0.25, 0.50 and 1 ng ml^{-1} for Cd and 6.25, 12.5 and 25 for Pb in electrothermal AAS. The assays were validated using certified samples of sediments (SD-M-2/TM IAEA) and mussel tissues (BCR, 278R). The total bioaccumulation of metals (Cd, Cu, Zn and Pb) in larvae was calculated by summing up the amounts that were measured in the soluble (S1) and insoluble (P1) fractions.

Assay of larval metallothionein.

The metallothionein assay was performed in the S2 fraction by differential pulse polarography. The thiol groups (SH) were determined using Brdicka reagent (1933) according to the method of Thompson and Cosson (1984). Measurements were performed at a constant

temperature (4°C) on a polarograph using a PAR Model 174 analyser, a PAR/EG&G Model 303 electrode in SMDE mode, and a RE0089 type X-Y reorder. The metallothionein amounts measured were determined by a standard addition using metallothionein rabbit liver metallothionein standard (Sigma Chemical Co., St Louis, MO, USA) (no metallothionein standard exists for oysters). The validity of this method was confirmed by Olafson and Olsson (1991).

Statistical analysis.

For each series of results, the comparison of values was tested by one-way ANOVA (Statistica software). Significant differences (at the 95% level) were then determined by Tukey's test, except for larvae exposed to 5, 10 and 25% of Dunkerque elutriate because there were only one value per tested elutriate concentration.

Results.

Sediment contamination

Metal concentrations in each sample of studied sediments are indicated in the Table 1. For comparison, Table 2 gives the French Géode classification system, which defines sediment quality according to the degree of contamination with PCBs and eight metals (Lamy 1996). "Géode background" denotes the natural concentrations, and "Géode median" is calculated from the samples taken at various ports in France. Sediments having values lower than double the median (termed "level 1") are allowed to be dumped offshore. Sediments having values higher than four times the median (termed "level 2") should not be dumped offshore. Sediments with intermediate values require further analyses, including bioassays, before a decision is taken. Dunkerque sediments were more highly contaminated than the Bidassoa ones. In the sediments from Dunkerque, Cd and Zn levels and Cu and Pb levels respectively

surpassed the level 1 and level 2. Bidassoa sediments were characterized by a Cu contamination with value higher than the level 1.

Larval biometry

Condition index (CI) of larvae exposed to Bidassoa elutriate was not significantly different depending on the exposure ($p = 0.274$; Table 3). For Dunkerque, the CI significantly ($p = 0.000064$) decreases between the control and the lowest concentration (1% elutriate). The CI of larvae exposed between 5 and 10% of elutriate was lower than of the CI of controlled ones. The CI of larvae exposed to 25% of Dunkerque elutriate was not indicated because the recovered larvae were only 5 days old.

Bidassoa elutriate induces a weak inhibition of the larval growth at the highest concentrations and on day 10 (Fig. 1), but the difference with the control value was not significant ($p = 0.07$). On the contrary, Dunkerque elutriate had deleterious effects on *C. gigas* growth (Fig. 1). The growth of larvae reared in 25% of elutriate was significantly reduced ($p = 0.0003$) from day 3 to the end of the experiment. The same phenomenon occurred at the all concentrations on day 5 ($p < 0.0006$).

Metallothionein concentrations

For Bidassoa elutriate, metallothionein contents in larvae significantly increase ($p = 0.0001$) for a concentration higher than 5 %. Metallothionein values ranged from 864 mg kg^{-1} for controls to 1,430 mg kg^{-1} in larvae exposed to 50% of elutriate (Fig. 2). The concentration ratio of exposed to control larvae ranged from 1.2 to 1.7 depending on the degree of contamination. For Dunkerque elutriate (Fig. 2), metallothionein concentration significantly increased between the control and the concentration attaining 1 % elutriate ($p < 0.05$), while the concentration rapidly decreased for the highest concentrations. The significant decrease of metallothionein between the control and concentration of 5, 10 and 25 could not be tested because of the lack of replicates. However, metallothionein concentrations in larvae exposed

to 5, 10 and 25 % elutriate were three or four times lower than those observed in larvae reared in control sea water and 1 % elutriate. Metallothionein concentration in 7 day-old control larvae (Dunkerque experiment) were significantly lower ($p < 0.0001$) than this in 10 days-old control larvae (Bidassoa experiment).

Metal bioaccumulation

Metal levels of larvae reared in the presence of the different elutriates were showed in figure 3. For the Bidassoa elutriate, no significant ($0.108 < p < 0.9938$) bio-accumulations of Cd, Zn and Pb was observed in larvae. Only Cu levels significantly ($p = 0.0001$) increased for elutriate concentration higher than 5%. The Cu concentration ratio of exposed to control larvae ranged from 1.1 to 1.8 depending on the degree of contamination. Larvae exposed to Dunkerque elutriate show contamination levels higher than those exposed to Bidassoa (except in Zn). Cd and Cu concentrations increased according to the elutriate concentration. However, the lack of replicates did not allow to test if this increase is significant. With Zn and Pb, significant ($p < 0.108$) accumulations were observed for the lowest tested concentration (1 %). The concentration ratio of exposed to control larvae were ranged from 1.6 and 2 for the Zn and from 2 to 20 for the Pb.

Cytosolic fractions showed similar contamination patterns than those previously observed in raw organism (Fig. 4), except for the Cd at the highest concentration of Bidassoa elutriate, where the accumulation was significant and for the Pb with the Dunkerque elutriate where accumulation was not observed.

Metallothionein is a cytosolic heat-stable protein. Consequently it should be preferable to examine the relationship between metal and metallothionein levels, taking into account metal concentrations in the supernatant S2 obtained after heat denaturation of the cytosol. (However, the fate of metals during heating is a matter of question, since metal analysis of chromatographic fractions obtained from raw cytosol (S1) and heat-denaturated cytosol (S2)

revealed differences in metal binding to cytosol ligands (Bragigand and Berthet, 2003)). Consequently, relationships between metallothionein and S1 metal concentrations were studied. As all studied elements could be binding to metallothionein and might therefore contribute concomitantly to metallothionein induction, the relationship between metallothionein and metal levels (S1) was examined taking into account the metals individually or combined. For relationship, all data were taking into account except with the highest elutriates concentration from Dunkerque (5, 10 and 25%) (Fig. 5), where deleterious effects are noticed (growth inhibition). Positive and significant relationships were observed for Cu, Zn and for all combined elements.

From a biomonitoring point of view and aiming to use metallothionein as a biomarker of metal contamination, metallothionein has to reflect the gross metallic concentration. Thus relationship between total metal (S1+P1) and metallothionein levels was equally examined. Results were similar than those previously observed with cytosolic metal concentrations. Relationships between metal and metallothionein concentrations were significant for the Cu and Zn and in the case of all elements combined (not shown).

Discussion

According to Geffard et al. (2002a), Bidassoa and Dunkerque sediments are heavily contaminated by metals. In comparison with the French Géode classification system, Dunkerque sediment is characterized by a Cu and Pb contamination and is more contaminated than the Bidassoa one which is only Cu-enriched. Dunkerque sediments are equally highly contaminated by polycyclic aromatic hydrocarbons (Geffard et al. 2002b).

Physical and chemical characteristics of the Bidassoa and Dunkerque elutriates have been shown in a previous paper (Geffard et al. 2002a). The metal contamination of the controlled

sea water was in the same range of magnitude as the metal levels observed in waters from uncontaminated areas (Campanella et al. 2001, Prego and Cobelo-Garcia 2004) and lower than those of the Gulf of Gaeta, Tyrrhenian Sea (central Italy), known to be slowly contaminated (Conti and Cecchetti 2003). Metal contents of the Bidassoa and Dunkerque elutriates were approximately 10 times more concentrated than the controlled seawater, with values in the same range of magnitude as metal contaminations currently observed in impacted coastal and estuary areas (RNO 1995, Martino et al. 2002). Cu levels in Bidassoa and Dunkerque elutriates exceeded environmental quality standard (Matthiessen et al. 1999). According to Slotten and Reuter (1995) and Van den Berg et al. (2001), these results showed that resuspending sediments induce an increase of the total seawater metal contamination.

Bio-availability of contaminants may be determined using bio-accumulation test. With Bidassoa elutriates, only the copper was bio-available and accumulated by the larvae. For Dunkerque, all studied metals were bio-available and accumulated by larvae. These results are in agreement with metal contamination levels of these sediments. However, highest metal contaminations (difference between control and exposed larvae) were observed in larvae exposed to the Dunkerque elutriates, whereas the contamination level of the highest tested concentration (25 %, that is to say 0.019µg $Cd.l^{-1}$, 1.78µg $Cu.l^{-1}$, 6.88µg $Zn.l^{-1}$ and 3.6 µg $Pb.l^{-1}$, estimated according metals concentration in raw elutriate published in Geffard et al. 2002a) was lower than the level of Bidassoa elutriates (50 %, 0.054µg $Cd.l^{-1}$, 5.65µg $Cu.l^{-1}$, 13.65µg $Zn.l^{-1}$ and 4.5µg $Pb.l^{-1}$). These observations show that bio-availability of metals from the Dunkerque elutriates was higher than those from Bidassoa one (Geffard et al., 2002a). According to Fichet et al. (1998) and Geffard et al. (2002a) parts of the heavy metals released in the water, when sediments are suspended again, and / or are bio-available, could be accumulated and toxic. According to Borgmann (2000), contamination level in larvae is a better indicator of potential biological impact than the contamination levels of medium.

In our previous study (Geffard et al. 2002a), no observed concentration effect of Bidassoa and Dunkerque elutriates on *C. gigas* embryogenesis was respectively 50 and 25 %. Bidassoa elutriate concentration of 50 % did not induce deleterious effects on the growth and condition index of larvae, after 10 day exposure. On the contrary, with Dunkerque elutriate larval growth test was highly more sensitive than the embryotoxicity test. Growth and CI inhibition were observed at the lowest elutriate concentration (1%). These results are in accord to those of His et al. (1999) who found that larval growth is the most sensitive life stage in this organism. The decrease of CI indicates that in exposed larvae, the inhibition of weight was more important than the inhibition of the height. CI constitutes an interesting and early warning marker of contaminant effects on larvae. These observations show that released sediment-bound contaminants may affect physiology and metabolism of oyster larvae. The re-suspending of sediments constitutes really a risk for pelagic species.

Larval growth test in *C. gigas* is a sensitive bioassay and allows to assess the toxicity of bio-available contaminants, constituting a useful tool to study the biological quality of sediments. However, they do not allow identifying the compounds inducing the observed biological effects.

Metallothionein level in larvae exposed to Bidassoa elutriates increased with increasing degree of contamination. Metallothionein is thought to be produced in response to increased intracellular levels of free metal to prevent or reverse potentially detrimental, non specific binding of metals to other ligands (Roesijadi et al. 1996). These observations confirm that released metals from Bidassoa sediment were really bio-available. Ringwood and Brower (1993, 1995) and Roesijadi et al. (1996) have also showed metallothionein inductions in *C. gigas* larvae exposed to metallic salts under laboratory conditions. However, they found that first induction occurred at a Cd concentration of 0.6 $\mu g.l^{-1}$, which is 30 fold higher than the Bidassoa elutriate concentrations of 10 % (0.018µg $Cd.l^{-1}$) that carry out a significant

metallothionein induction in this study. The precocity of metallothionein response to metals bioaccumulation, according to the degree of exposure, compared to the responses of other physiological endpoints taken into account in this study (growth and CI) authorize to define a scale of sensitivity of biological and biochemical responses in *C. gigas* larvae:

Metallothionein>larval growth = CI>abnormal embryogenesis

Similar observations were made by several authors in an other species of bivalve, *Mytilus galloprovincialis* (Pavicic et al. 1994, Geffard et al. 2002c).

For Dunkerque elutriates, metallothionein levels slowly increased between the control and the elutriate concentration of 1%, and then highly decreased. These observations could be explained by the poor physiological state of larvae (condition index and growth lower than controls). These results are good examples of a situation which have previously been mentioned as 'spillover' (Brown and Parsons 1978). This is the saturation of detoxification mechanisms, carrying out the first harmful effects. This phenomenon has been described in several species in laboratory, the fish *Pleuronecta platessa* (Georges and Olsson 1994), copepods, *Tigriopus brevicornis* (Barka et al. 2001) and bivalve larvae *Mytilus galloprovincialis* (Geffard et al. 2002c).

According to the previous results and the good relationships observed between the most bio-accumulated metals (Cu and Zn) and metallothionein concentrations indicated that metallothionein level reflect : 1 - the cytosolic metal charge was the most toxicologically important (Wallace et al. 2003) , 2 - the gross metallic concentrations (S1+P1) suggested the possible use as metal exposure biomarker. The absence of relationship between metallothionein and cadmium concentrations could be explained by the very low bioaccumulation of this metal by larvae. Concerning the lead, the total bio-accumulated metal due to the elutriate exposure was found in the insoluble fraction (P1), suggesting a poor implication of metallothionein in the detoxification of this element and equally a low

induction of metallothionein synthesis by lead. As shown with other species (*Mytilus edulis* larvae, Geffard et al. 2002c) oyster larvae could be used as biological matrix to determined metallothionein as biomarker of metallic pollution. The highest advantage to use the larvae stage of bivalve as biological matrix compared to adult stage, is due to the higher sensitivity of this stage to different contaminants family (His et al. 1999). The potential use of the determination of metallothionein as biomarker in bivalve larvae in biomonitoring programs could be realised during *in situ* tests as developed by Geffard et al (2001b).

Larvae exposed to the highest Dunkerque elutriate concentrations (5, 10 and 25%) had the highest metal body burden and corresponded to the lowest metallothionein level. These individuals constitute "fault controls" not due at low contaminants levels exposure, but inversely at very high contaminants level exposure, which induce detrimental effects and the phenomenon of spill over as described above. In biomonitoring programs, in order to avoid these fault controls, it is important to use several biochemical biomarkers more or less specific to a contaminants family as proposed by several authors (Narbonne et al. 1999, de Lafontaine et al. 2000, Cajaraville et al. 2000) including easily measurable global physiological markers as condition index.

These results showed that Dunkerque sediments constitute a biological hazard when they are suspended again (example: dredging). Moreover the present study, using metal concentrations similar to those found in the natural environments, showed an early (low-dose) metallothionein response as compared to the time that abnormalities appeared. Thus, the determination of metallothionein in oyster larvae could be use as biomarker of metal exposure, which represents an important ecological factor because the successful breeding is necessary to maintain the population.

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Figure captions

Figure 1: Growth (µm; mean ± SD) of *Crassostrea gigas* larvae exposed to Bidassoa (A) and Dunkerque elutriates (B) for ten and seven days, respectively. Statistical comparison is presented in the text.

Figure 2: Metallothionein contents (mean ± SD, n = 3) in larvae after ten days (Bidassoa, white bars) or seven days (Dunkerque, black bars, except at 25% only 5 days) of exposure to different elutriate concentrations. For Bidassoa, values not significantly different from each other are grouped under a common overhead line (ANOVA, $p < 0.05$; Scheffé's test). For Dunkerque only control larvae and larvae exposed to 1% of elutriate could be compared (see text) and * indicate a significant difference at 95% level.

Figure 3. Total metal concentrations (mean ± SD, three replications) in larvae after ten days (Bidassoa, white bars) or seven days (Dunkerque, black bars; except at 25% only 5 days) of exposure to different elutriate concentrations. A, Cd; B, Cu; C, Zn and D, Pb. For statistical comparison presentation, see Figure 2 legend.

Figure 4. Cytosolic metal concentrations (mean ± SD, three replications) in larvae after ten days (Bidassoa, white bars) or seven days (Dunkerque, black bars; except at 25% only 5 days) of exposure to different elutriate concentrations. A, Cd; B, Cu; C, Zn and D, Pb. For statistical comparison presentation, see Figure 2 legend.

Figure 5. Relationships between cytosolic (S1) metal (individual or combined) and MT concentrations in whole larvae categories (A, Cd; B, Cu; C, Zn; D, Pb and E, Cd+Cu+Zn+Pb). The linear regression take into account all larvae excepted larvae showing dramatic biological effects (larvae exposed to 5, 10 or 25% of Dunkerque elutriate). Significant correlations (at 95% level) are indicated with an asterisk.

List of tables

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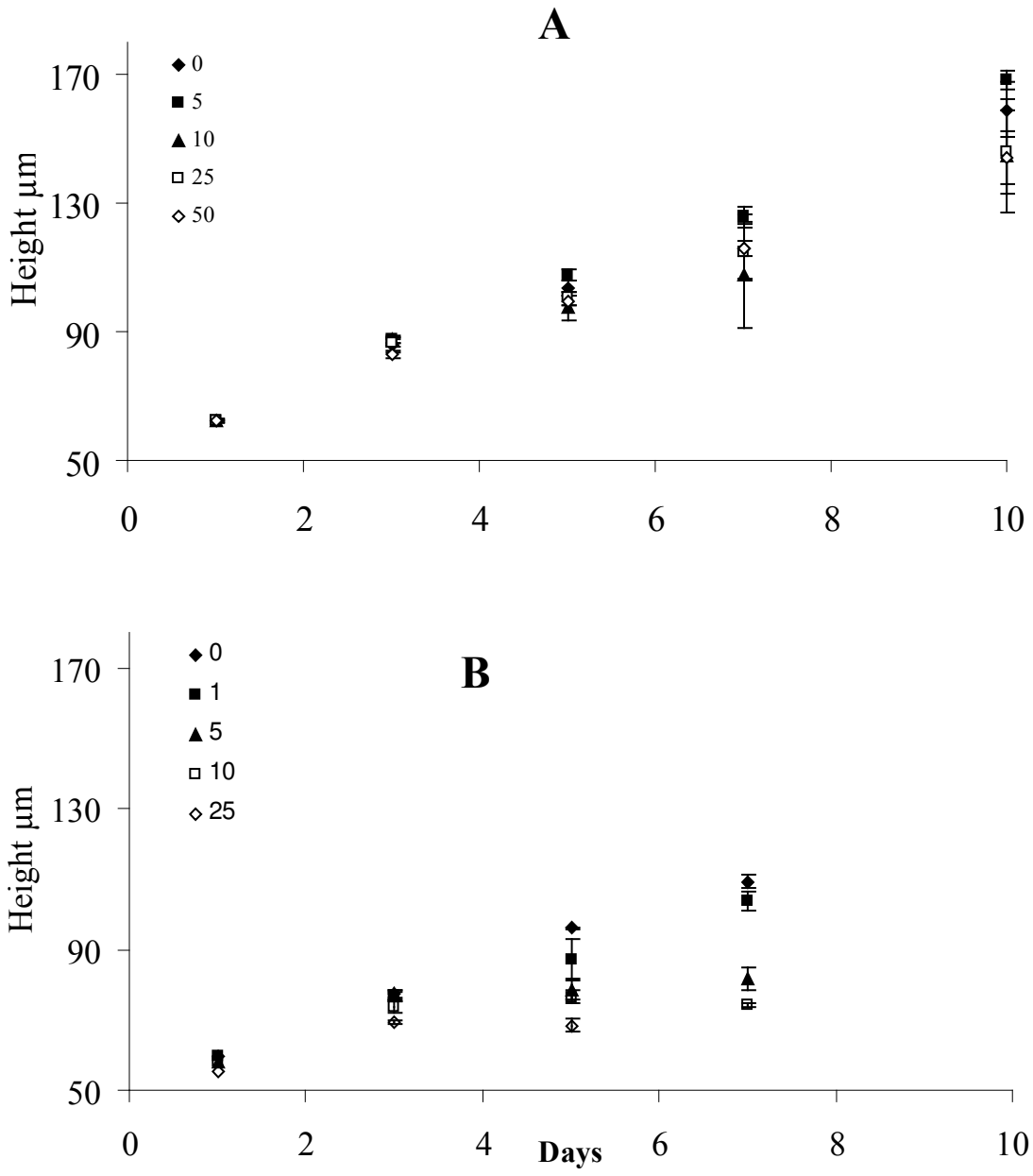

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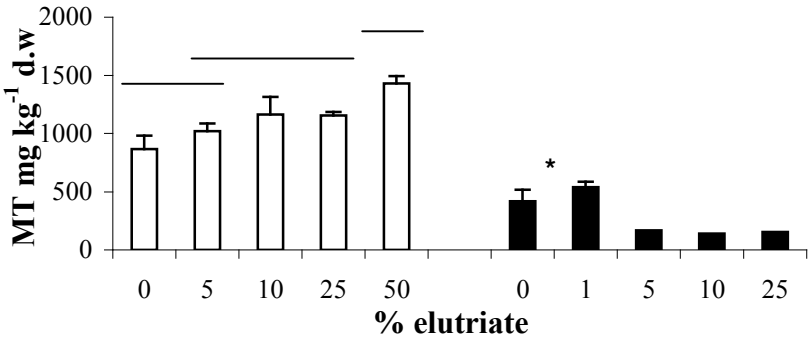

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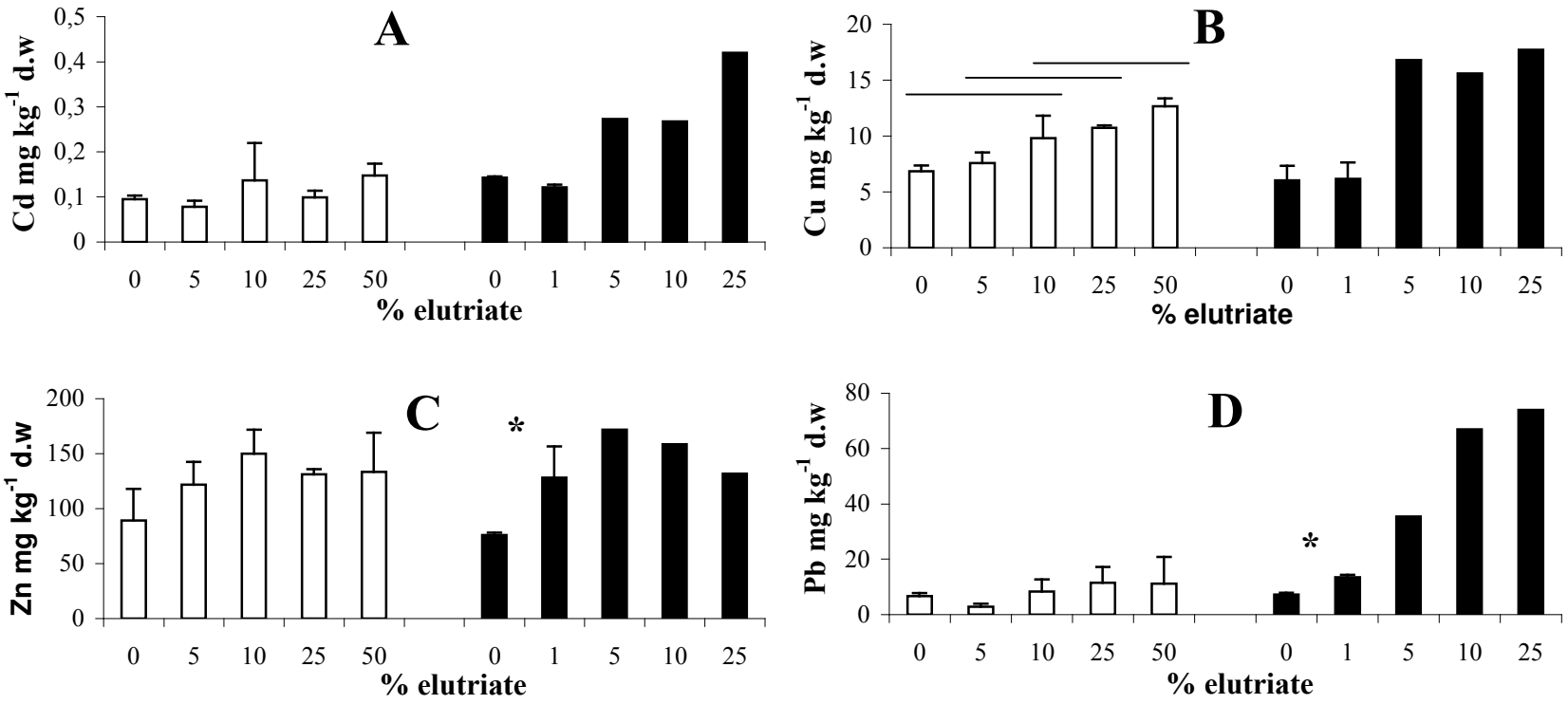

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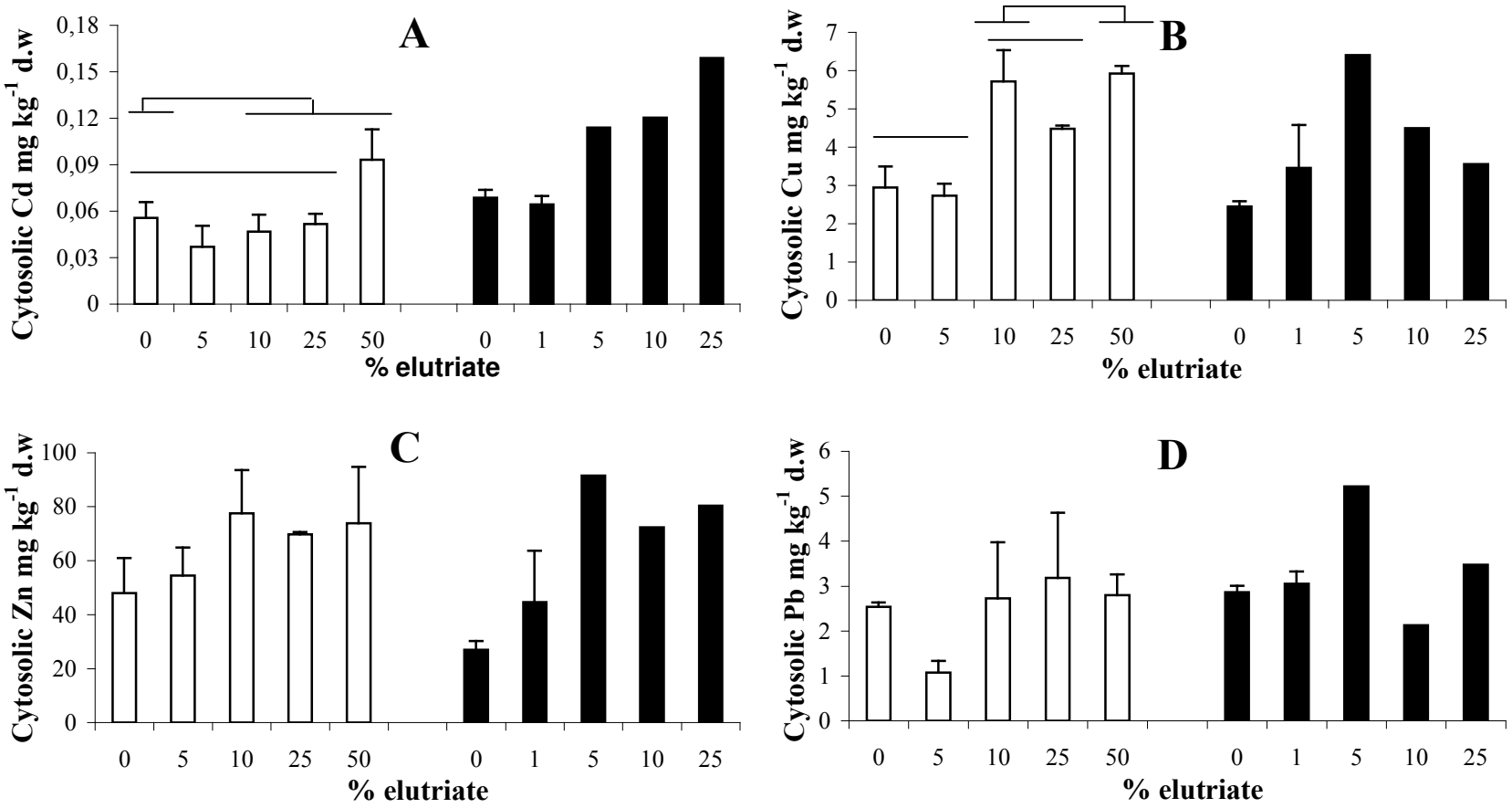

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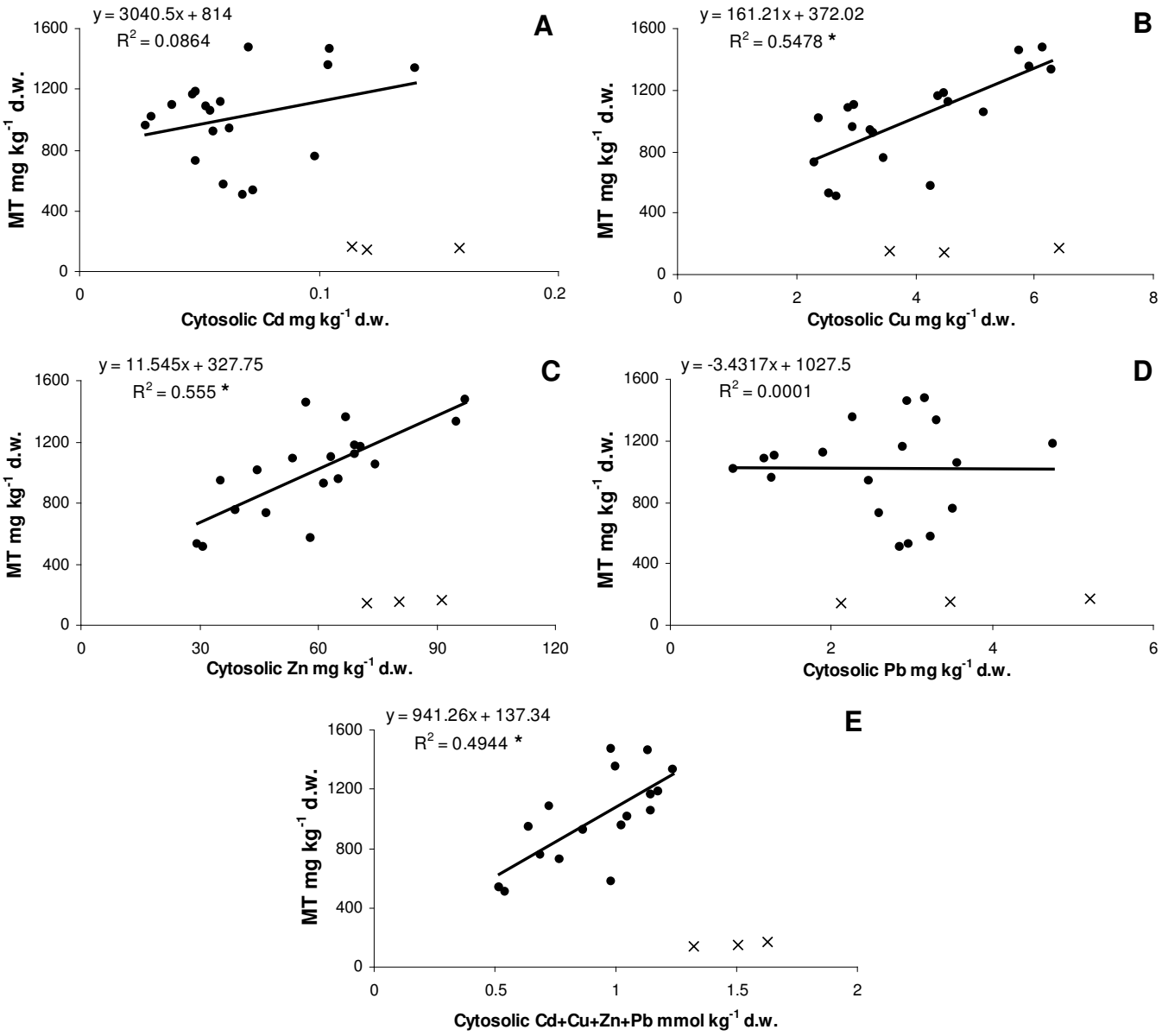

Table 1. Heavy metals (mean μg g^{-1} dry weight ± SD) contents of Dunkerque and Bidassoa sediments.

Site	Cd	Cu	Zn	Pb
Dunkerque	2.2 (0.05)	158 (10)	542 (36)	391 (24)
Bidassoa	0.8 (0.1)	70 (11)	268 (31)	74 (9)

Table 2. French "Géode" classification of sediment quality (Md = median of metal concentrations µg g^{-1} dry weight).

	PCB[a]	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
Géode background	0	4.4	0.5	45	35	0.2	20	47	115
Géode median	0.00025	12.5	0.6	45	22.5	0.2	18.5	50	138

[a] PCB = polychlorinated biphenyl

Table 3. Condition index (CI, mean; ± SD between bracket)of larvae recovered after 10 (Bidassoa) or 7 (Dunkerque) days of rearing in medium containing different percentage of elutriate. No CI was calculate in the case of 25% of Dunkerque elutriate because rearing was stopped after 5 days. * indicate a significant difference at 95% level of the CI compare to the control (0%) for each sediment tested

Site	Parameter	0%	1%	5%	10%	25%	50%
Bidassoa (Day 10)	CI	0.52 (0.10)		0.68 (0.09)	0.44 (0.31)	0.50 (0.17)	0.46 (0.08)
Dunkerque (Day 7)	CI	0.39 (0.006)	0.24* (0.02)	0.16* (0.0008)	0.23* (0.006)	/	